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Interactions between retinoic acid, nerve growth factor and sonic hedgehog signalling pathways in neurite outgrowth

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Abstract

Many studies have shown a role of retinoid signalling in neurite outgrowth in vitro, and that the retinoic acid receptor (RAR) β2 is critical for this process. We show here that RARβ2 is expressed predominantly in dorsal root ganglia (DRG) neuronal subtypes that express neurofilament (NF) 200 and calcitonin gene-related peptide (CGRP), and that these neurons extend neurites in response to RA. We demonstrate that retinoid signalling has a role in neurite outgrowth in vivo, by showing that in a peripheral nerve crush model there is less neurite outgrowth from RARβ null DRG compared to wild-type. We identify sonic hedgehog (Shh) as a downstream target of the RARβ2 signalling pathway as it is expressed in the injured DRG of wild-type but not RARβ null mice. This regulation is direct as when RARβ2 is overexpressed in adult motoneurons Shh is induced in them. Finally we show that Shh alone cannot induce neurite outgrowth but potentiates RARβ2 signalling in this process.

Keywords: Retinoic; Regeneration; Neurite

Introduction

The retinoid-signalling pathway has been shown to be involved in neurite outgrowth (Quinn and De Boni, 1991) mediated through the retinoic acid receptor (RAR) β2 (Corcoran and Maden, 1999; Corcoran et al., 2000; Dmetrichuk et al., 2005). RARβ2 mediates gene transcription by heterodimerising to a retinoid X receptor (RXR) and binding to a retinoic acid response element (RARE) in the target genes (Bastien and Rochette-Egly, 2004). Transcription occurs once a retinoid interacts with the RAR/RXR heterodimer (Bastien and Rochette-Egly, 2004).

In the peripheral nervous system, the DRG consists of several types of neurons that can be identified by their expression of various markers. These are heavy neurofilament protein (NF200), the neuropeptide calcitonin gene-related peptide (CGRP) and isolectin B4 (IB4), which bind large-diameter myelinated, small-diameter unmyelinated peptidergic and non-peptidergic classes, respectively (Averill et al., 1995; Bradbury et al., 1998; Lawson et al., 1984).

It is not yet known if RARβ2 signalling is required for neurite outgrowth of all subtypes of DRG neurons or the genes regulated by this signalling pathway. One downstream target of RARβ signalling involved in neurite outgrowth may be sonic hedgehog (Shh), which has a RARE in its promoter (Chang et al., 1997). It can act as a chemoattractant for commissural axons (Charron et al., 2003) and is induced in response to motoneuron injury, where it has been shown to play a role in neuronal survival (Akazawa et al., 2004).

We have compared the neurite regenerative response of gene deleted RARβ and wild-type mice in vitro and in vivo in order to explore the role of retinoid signalling in neurite outgrowth from adult DRG. We show that NF200- and CGRP-expressing DRG neurons are dependent upon RA signalling for neurite outgrowth. We further show in a peripheral nerve injury model that there is

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less axonal regeneration in the RARβ null than in the wild-type mouse, and that one of the downstream signalling pathways is Shh and that it enhances RARβ2-mediated neurite outgrowth.

Materials and methods

DRG cultures

Whole DRG were isolated from the cervical region of adult RARβ2 null mice (Mendelsohn et al., 1994) and wild-type mice. They were then placed in a cellogen matrix (ICN flow) and cultured for three days in serum-free medium in the presence of the appropriate factor. Serum-free medium consisted of DMEM-F12 (Invitrogen), 2 mM glutamine (Invitrogen), 33 mM glucose (Invitrogen) and ITS-A (Invitrogen). The factors used were 0.1 μM all-trans RA (Sigma), 20 ng/ml NGF (Promega) and 20 ng/ml GDNF (Promega), and 10 μm cyclopamine (Gift from H. Blidscoe). Shh-containing conditioned medium was obtained from cultures of QT6 cells overexpressing shh cDNA as previously described (Li et al., 2004). Neurites were traced, counted and measured using image pro plus system. Graphs were plotted and statistics carried out using Sigma Plot/Stat software. Histograms show mean ±SEM of either number or average length of neurites (μm) from 9 explants.

In situ hybridisation and immunohistochemistry

Both in situ hybridisation and immunohistochemistry were carried out as previously described (Corcoran et al., 2000). The antibodies used were obtained from Sigma except where stated. These were, anti-mouse N52, which detects NF200 (used at 1:200 dilution), anti-rabbit CGRP (used at 1:200 dilution) and the isolectin IB4 (used at 1:100 dilution). Secondary antibodies used were anti-rabbit Cy3 conjugated (Jackson, used at 1:1000) AlexaFluor™ 633 (Molecular probes, used at 1:1000 dilution) and extra Avidin-FITC conjugated (used at 1:500 dilution). For DRG explants, triplicate staining was performed. For in situ hybridisation, fresh frozen tissue was used. In total, 3 C4 DRG from three different animals were obtained. Twelve-micrometer sections were obtained. The in situ was performed and developed first and then the immunohistochemistry with either N5.2 (on sections 1, 4, 7 and 10), CGRP (on sections 2, 5, 8 and 11) or IB4 (on sections 3, 6, 9 and 12) was carried out on the developed slides. Using Image-Pro Plus software, the total number of positive neurons per section for each neuronal subtype marker was obtained, and then the total number of neurons expressing a neuronal subtype marker and RARβ. Graphs were plotted showing percentage of positive RARβ neurons for a particular neuronal subtype and statistics carried out using Sigma Plot/Stat software.

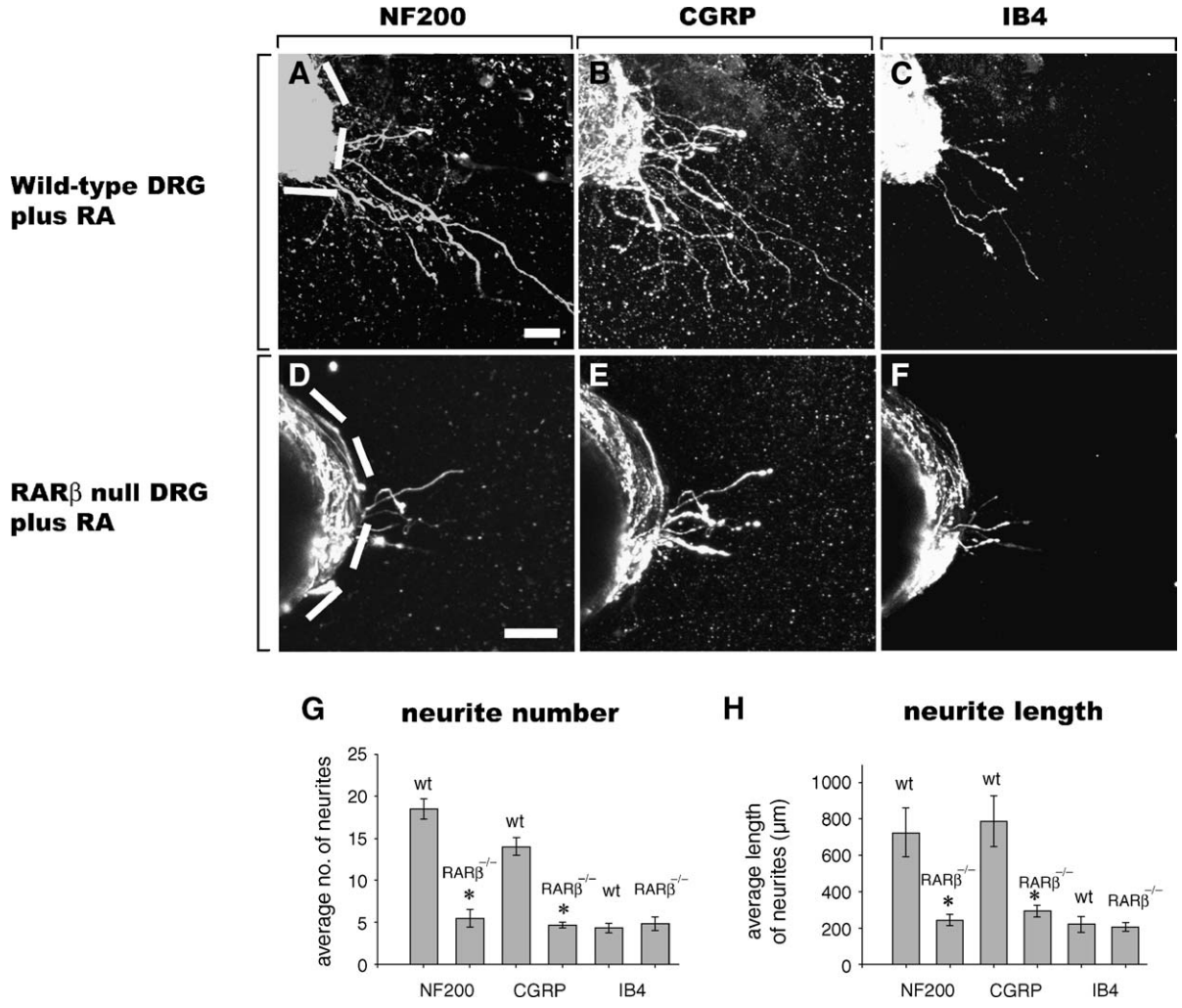

Fig. 1. Effect of RA on neurite outgrowth from cultured adult DRGs from wild-type and RARβ2 null mice. Adult DRG were cultured in a cellogen matrix in serum-free medium plus 0.1 μm RA for 3 days and immunohistochemistry carried out with the three neuronal markers, NF200, CGRP and IB4. (A–C) Wild-type DRG. (D–F) RARβ null DRG. (G, H) Quantification of number of neurites and their lengths. RA induces the regeneration of CGRP and NF200 expressing neurons in the wild-type but not RARβ2 null mouse whereas there is no effect on IB4 expressing neurons. *$P<0.05$, Student's t test. White lines denote edge of explant. Scale bar: 50 μm.

Sciatic nerve crush and transduction of lentiviral constructs

The experiments were undertaken in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Adult RARβ null ($n=5$) and wild-type ($n=6$) mice were anaesthetised with isoflurane. The left sciatic nerve was exposed and was completely crushed for 15 s with fine forceps at the mid-thigh level, the injury site was marked with Indian Ink. Three days later, the mice were overdosed with sodium pentobarbitone and perfused transcardially with 0.9% NaCl solution containing heparin followed with 4% paraformaldehyde in 0.1 M phosphate buffer. The sciatic nerve was removed and transferred to 20% sucrose and then embedded in OCT compound. Ten-micrometer longitudinal sections were cut on a cryostat and mounted onto superfrost slides. The immunohistochemistry was carried out using anti-GAP43 (1:2000, gift from G. Wilkin). Secondary antibody used was AlexaFluor™ 488 (Molecular Probes, used at 1:1000 dilution). Quantitative analysis of axonal regeneration was carried out by counting individual GAP43-positive axons at measured intervals with a 1-mm square grid graticule proximal and distal to the nerve crush site. At least 3 longitudinal sections per animal were counted.

The construction and preparation of the Lentiviral constructs were carried out as previously described (Corcoran et al., 2002). Adult rats, $n=3$ for each construct, were anaesthetised as above. The spinal cord at the level of C4 was exposed and transduced with 3 μl of the appropriate virus using an osmotic mini-pump. Five weeks after transduction the animals were sacrificed and the spinal cord tissue processed and in situ hybridisation performed as above.

Results

RARβ signalling is required for neurite outgrowth in the peripheral nervous system

In order to ask if RARβ2 is essential for neurite outgrowth of the NF200, CGRP and IB4 expressing subtypes of DRG neurons, we cultured adult DRGs from the cervical region in a cellogen matrix from wild-type and RARβ2 null mice in the presence of serum-free medium plus 0.1 μM RA. After 3 days, we assayed for neurite outgrowth using the three neuronal markers NF200, CGRP and IB4. Neurite outgrowth could be detected with NF200 (Figs. 1A and D), CGRP (Figs. 1B and E) and IB4 (Figs. 1C and F) in DRGs cultured from wild-type and RARβ null mice. However, the numbers of NF200 and CGRP

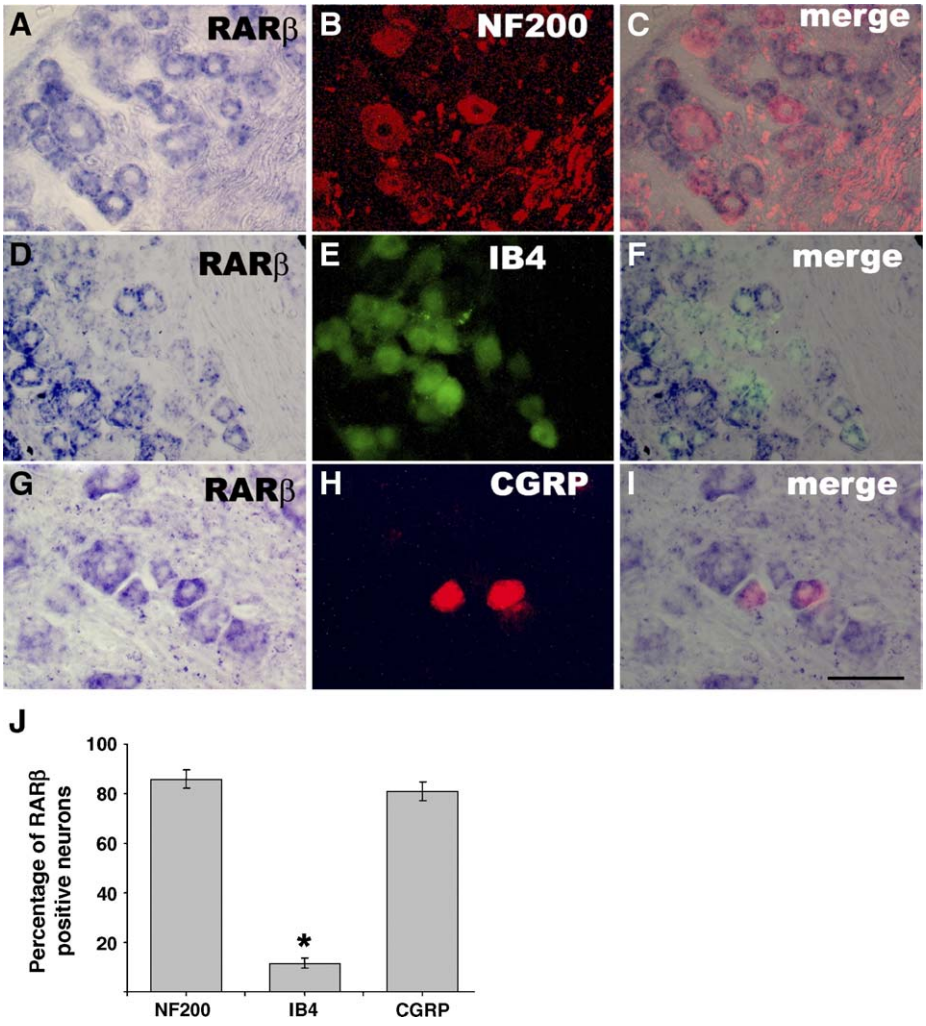

Fig. 2. Expression of RARβ2 in subsets of adult DRG neurons. In situ hybridisation of RARβ and immunohistochemistry of either NF200, CGRP or IB4. (A–C) Expression of RARβ and NF200. (D–F) Expression of RARβ and IB4. (G–I) Expression of RARβ and CGRP. (J) Quantification of number of NF200, CGRP and IB4 neurons expressing RARβ2. The majority of NF200 (85%) and CGRP (80%) neurons express RARβ2, whereas significantly less IB4 neurons (12%) express this receptor. *$P<0.005$, Student's t test. Scale bar: 50 μm.

neurites were significantly greater and longer from wild-type than RARβ2 null DRG, but there were no significant differences in their number and lengths with respect to IB4 (Figs. 1G and H). These data suggest that the RARβ2 receptor mediates the retinoid-signalling response for neurite regeneration and that it is limited to neurons that predominantly express NF200 and CGRP. By in situ hybridisation, 85% of the NF200 (Figs. 2A–C, J), 12% of IB4 (Figs. 2D–F, J) and 80% of CGRP neurons expressed the receptor (Figs. 2G–J). This suggests that the RARβ2 signalling pathway is mediated through a direct effect on the neurons themselves.

Effect of neurotrophins on neurite outgrowth of DRG explants from wild-type and RARβ null mice

We next asked whether there was a defect in neurotrophin-dependent outgrowth in the RARβ null DRG. Adult DRG from wild-type and RARβ2 null mice were cultured in the presence of NGF or GDNF and analysed for neurite

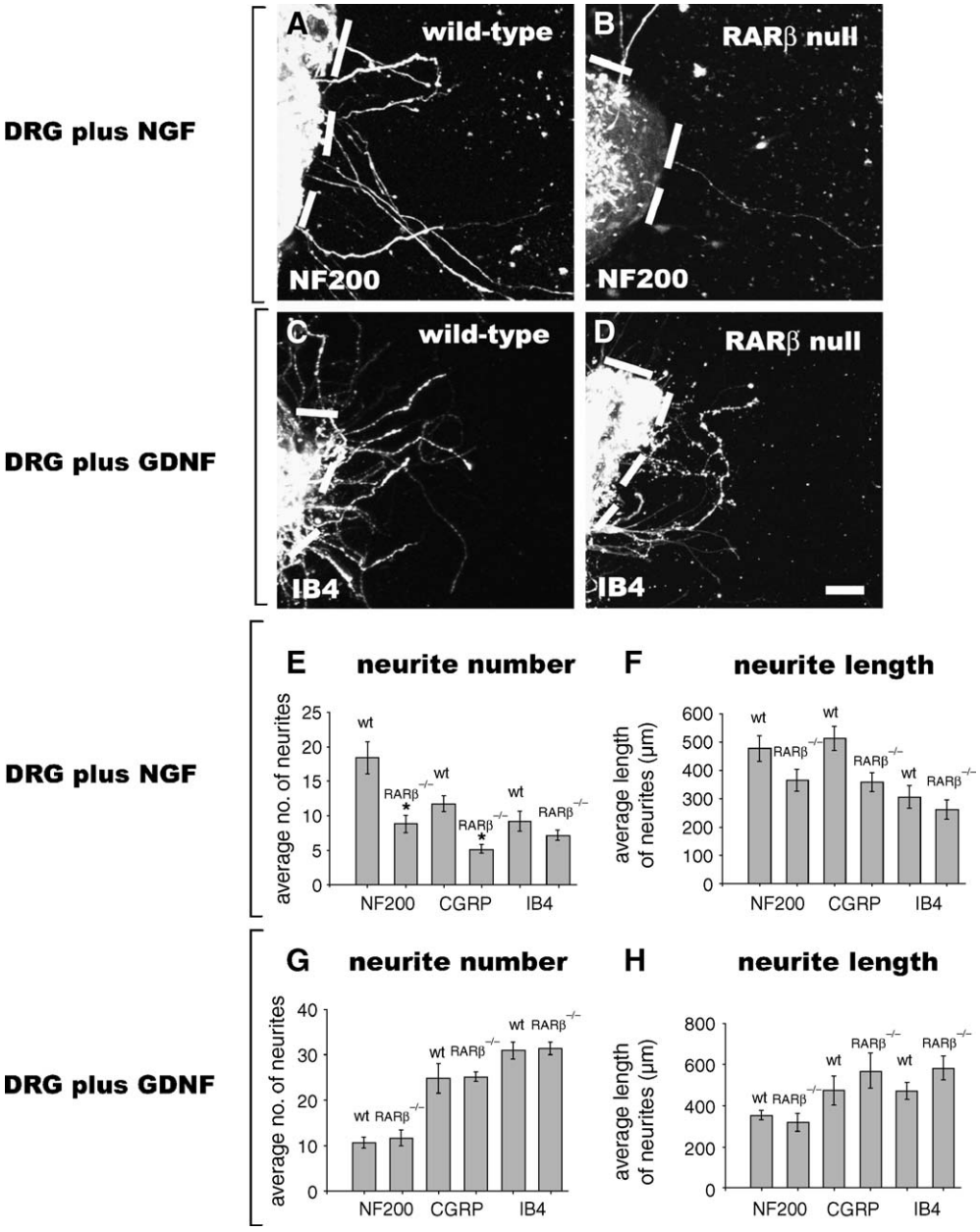

Fig. 3. Effect of neurotrophins on neurite outgrowth from cultured adult DRGs from wild-type and RARβ2 null mice. Adult DRG were cultured in a cellogen matrix in serum-free medium plus 20 ng/ml of either NGF (A, B) or GDNF (C, D) for 3 days. Immunohistochemistry of NF200 and IB4. (A, C) Wild-type DRG. (B, D) RARβ2 null DRG. (E–H) Quantification of number of neurites and their lengths. There is a deficiency in NF200 and CGRP neurite outgrowth in response to NGF in RARβ null compared to wild-type DRG whereas in response to GDNF the two types of DRG behave similarly. *$P<0.05$, Student's t test. White lines denote edge of explant. Scale bar: 50 μm.

outgrowth as described above. In the presence of NGF, neurites could be detected from both wild-type and RARβ null DRG expressing predominantly NF200 (Figs. 3A and B) and CGRP and from some neurons expressing IB4 (data not shown). The average length of neurites from wild-type DRG in response to NGF was 500 μm for both NF200 and CGRP neurons (Fig. 2F), compared to 800 μm for RA-stimulated DRG (Fig. 1H) although their numbers were approximately the same (Figs. 1G and 2E). There were significantly less NF200- and CGRP-positive neurites, but their lengths were not significantly different, in DRG from RARβ null mice compared to wild-type mice (Figs. 3E and F). In the presence of GDNF, the majority of neurites from wild-type and RARβ null DRG were mainly IB4 positive (Figs. 3C and D) and CGRP (data not shown) positive; however, there was no significant difference in either their numbers or length between the RARβ null and wild-type DRG (Figs. 3G and H).

In order to eliminate the possibility that the RARβ null DRG may have a different neuronal composition to the wild-type mice, we compared the number of NF200, IB4 and CGRP expressing neurons. There was no difference in the number of neuronal subtypes between the wild-type and RARβ null DRG at the level of C4 (data not shown). The fact that there is a deficiency of neurite outgrowth in the NGF-stimulated DRG in the RARβ null compared to the wild-type mouse suggests that RARβ interacts with the NGF signalling pathway.

Peripheral nerve regeneration is impaired in RARβ null mice and this correlates with a defect in sonic hedgehog signalling

We next asked if RARβ2 deficiency resulted in a defect in peripheral nerve regeneration in vivo. The peripheral nerve was crushed and the amount of axonal regeneration assessed three days later using GAP43. At increasing distances from the crush site there were significantly less regenerating axons in the RARβ2 null compared to the wild-type mice (Figs. 4A–C), suggesting that RARβ2 signalling is required for axonal extension in vivo.

It has recently been shown that Shh can act as a chemoattractant for commissural axons (Charron et al., 2003) and is induced in response to facial motoneuron injury (Akazawa et al., 2004). Therefore, we asked if Shh was involved in peripheral axonal regeneration. At three days after nerve injury, Shh mRNA was found to be induced in the neuronal cell bodies. However, its level of expression was much less in the RARβ null compared to the wild-type mouse, which suggests that RARβ2 may regulate Shh expression (Figs. 5A and B). In order to confirm this, rat cords that do not express RARβ2 (Corcoran et al., 2002) were transduced in vivo with

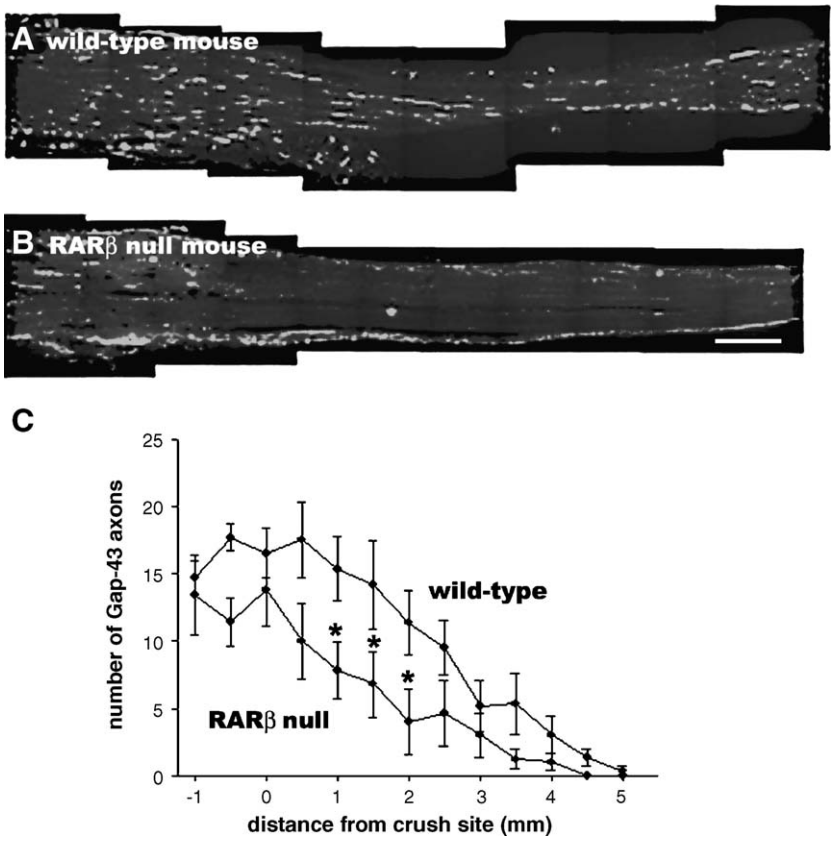

Fig. 4. Comparison of peripheral nerve regeneration between wild-type and RARβ2 null mice. The peripheral nerve was crushed and the regenerating axons were identified after three days with GAP43. Positive axons for GAP43 could be detected in (A) wild-type and (B) RARβ2 null mice. (C) At increasing distances from the crush site, there were significantly less regenerating axons in the RARβ null compared to the wild-type mice. *$P<0.05$, Student's t test. Scale bar: 1 mm.

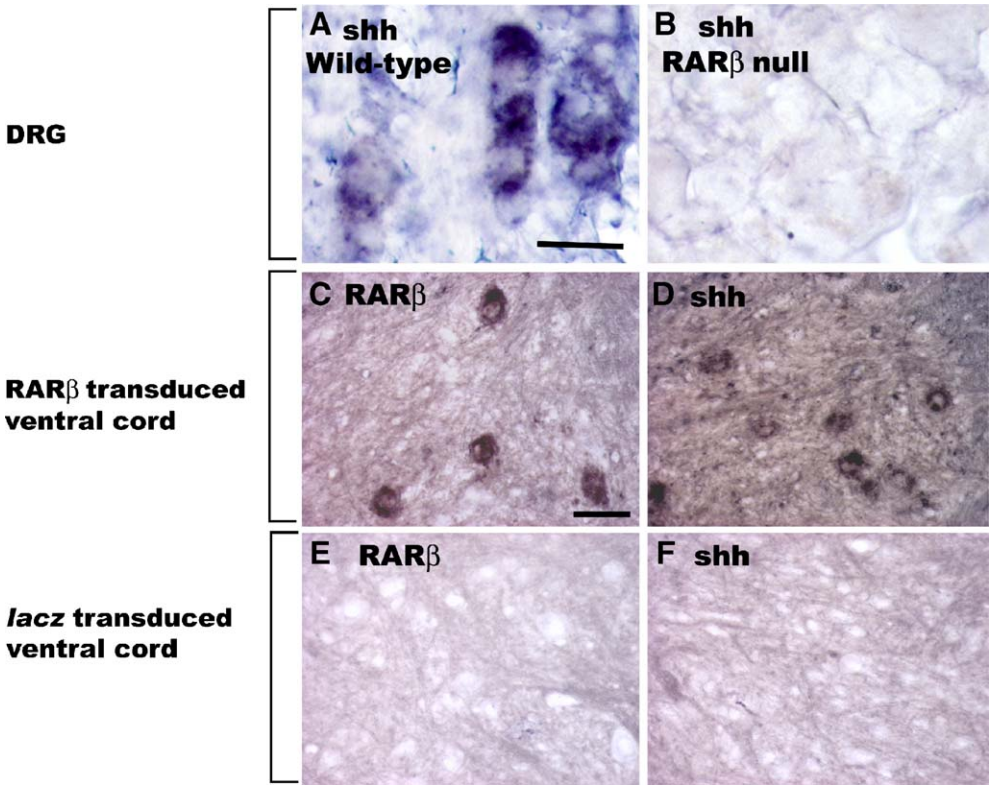

Fig. 5. Sonic hedgehog can be induced by peripheral nerve injury and by overexpression of RARβ2 in the rat spinal cord. Shh expression in the injured neurons of (A) wild-type mice (B) RARβ2 null mice. Lentiviral vectors expressing either RARβ2 or LacZ were transduced in vivo into adult rat spinal cord and in situ hybridisation carried out after five weeks. In RARβ2 transduced cords, RARβ2 (C) and Shh (D) can be detected in the motoneurons, whereas in the LacZ transduced cord neither RARβ2 (E) or Shh (F) are expressed. Scale bar: 20 μm in panels A and B and 20 μm in panels C–F.

lentiviral vectors expressing either RARβ2 or LacZ. Five weeks after transduction, we assayed for RARβ2 and Shh expression by in situ hybridisation. RARβ2 transduced cords showed both RARβ2 and Shh expression mainly in the motoneurons (Figs. 5C and D) whereas neither of these transcripts could be detected in the LacZ transduced cords (Figs. 5E and F). This finding suggests that the retinoid-signalling pathway acting via RARβ2 can induce Shh expression.

Sonic hedgehog does not induce neurite regeneration but potentiates it, when RARβ2 is activated

We next addressed the role of Shh in neurite regeneration by using media obtained from QT6 cells transduced with Shh cDNA, which secrete this protein. We first asked if Shh could substitute for RARβ2 signalling in neurite outgrowth. Adult DRG were cultured as above in the presence of Shh, after 3 days little or no neurite outgrowth could be detected with the three neurite markers NGF, CGRP and IB4 (Figs. 6A–C). Similarly cultured adult spinal cord explants, which do not express RARβ2 (Corcoran et al., 2002), could not regenerate in the presence of Shh (data not shown). This suggests that Shh is not sufficient to cause neurite regeneration even though it is downstream of the RARβ2 pathway. Therefore, we asked if Shh could potentiate RARβ2-mediated neurite regeneration. Adult DRG were cultured in the presence of RA and Shh. After 3 days there was abundant neurite outgrowth from NF200- and CGRP-positive neurons (Figs. 6D and E), which was greater than with RA alone (compare with Figs 1A and B), but there was little response from IB4-positive neurons (Fig. 6F). We next asked if the effect of Shh was mediated by its signalling component smoothened (Smo) by inhibiting it with cyclopamine (Chen et al., 2002; Frank-Kamenetsky et al., 2002; Incardona et al., 1998). When DRG were cultured in the presence of RA, Shh and cyclopamine the enhancement in NF200 and CGRP neurite outgrowth induced by Shh was abolished, whereas there was no effect on IB4 neurite outgrowth (Figs. 6G–I). There were significantly more and longer CGRP and NF200 neurites in the RA plus Shh-treated cultures than in the RA plus Shh and cyclopamine cultures (Figs. 6J and K).

Discussion

RARα, β and γ and their various isoforms are expressed in DRG neurons (Corcoran et al., 2000); however, of these it is RARβ2 that is essential for peripheral neurite regeneration in vivo as we have shown here that in RARβ2 null mice this process is impeded. Both RA and NGF signalling stimulates neurite outgrowth of NF200 and CGRP neuronal subtypes (Fig. 7) and it is these that predominantly express RARβ2. On the other hand, GDNF signalling stimulates neurite

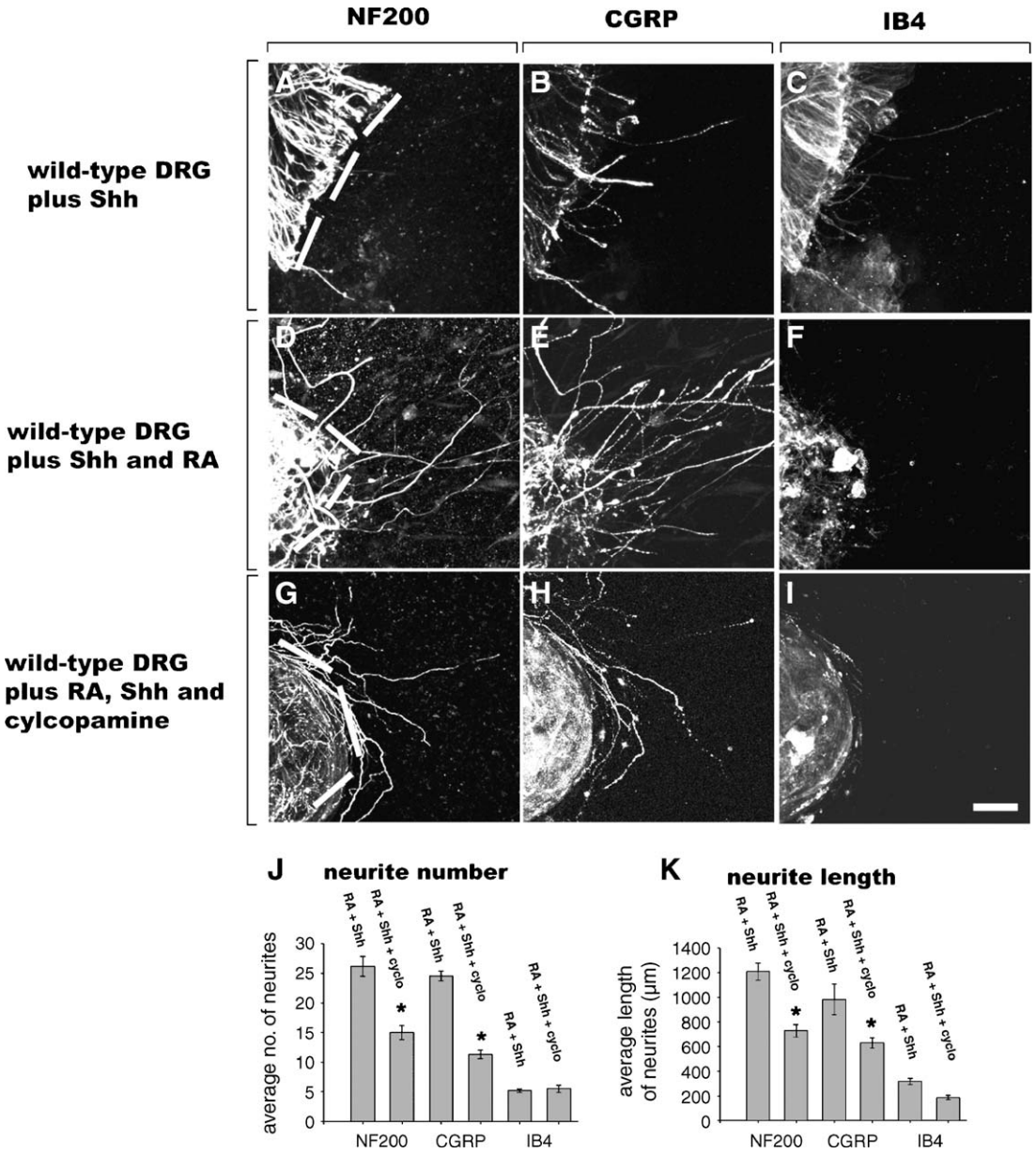

Fig. 6. Shh protein potentiates neurite outgrowth in the presence of RARβ2 signalling. (A–C) Shh in the absence of RA signalling cannot induce neurite outgrowth in wild-type DRG. (D–F) Shh potentiates neurite outgrowth in the presence of RA in NF200 and CGRP neuronal subtypes but not IB4 neuronal subtype. (H–J) In the presence of cyclopamine, Shh and RA the amount of neurite outgrowth is decreased from the NF200 and CGRP neurons to that of RA alone, whereas there is no effect on IB4 neurons. (K, L) Quantification of number of neurites and their lengths. *$P<0.05$, Student's t test. White lines denote edge of explant. Scale bar: 50 μm.

outgrowth of IB4 subtype neurons, the majority of which do not express RARβ2, nor extend neurites in response to RA (Fig. 7). This suggests that the action of RA on neurite outgrowth is predominantly a direct effect on the neurons themselves.

NGF signalling can upregulate RARβ2 expression (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001). This allows further upregulation of RARβ2 expression as the RARβ2 promoter contains a RARE that can bind the activated receptor (Leid et al., 1992). In the absence of RARβ2 signalling, NGF can still stimulate some neurite outgrowth (Fig. 7 and Corcoran and Maden, 1999), suggesting that NGF is involved in non-retinoid-mediated neurite outgrowth pathways.

RARβ signalling can also act independently of the NGF signalling pathway in neurite regeneration

There are thought to be two phases of neurite outgrowth, which rely on different patterns of gene expression (Smith and Skene, 1997). The initial phase consists of highly branched neurites with limited extensions; this appears to be dependent on NGF, both in vitro and in vivo (Diamond et al., 1987; Gavazzi et al., 1999). The later phase, where a transcriptional

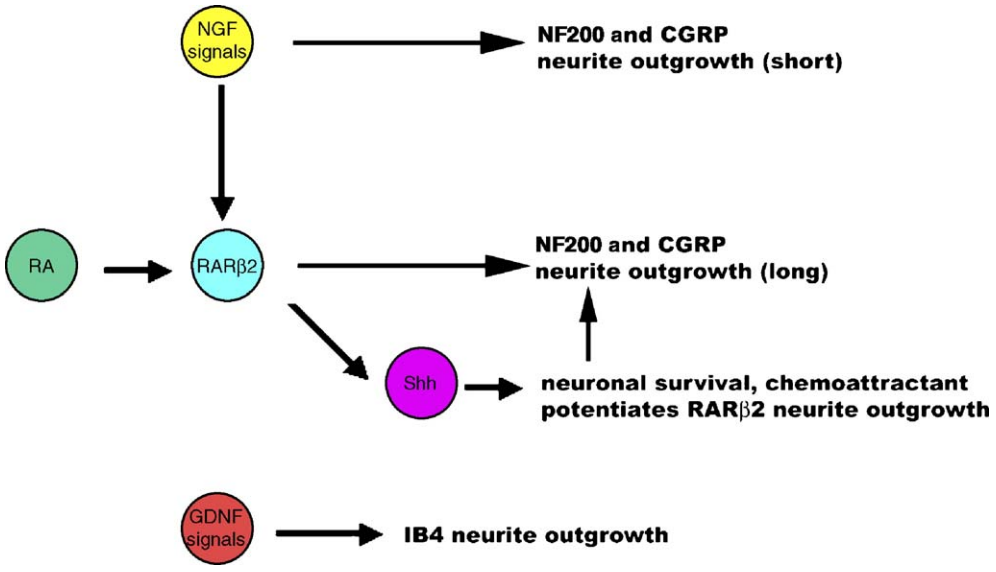

Fig. 7. Interaction of RARβ signalling with other signalling pathways in neurite outgrowth. NGF signalling initiates neurite outgrowth of NF200 and CGRP expressing neurons, this pathway can induce RARβ2 (Corcoran and Maden, 1999; Cosgaya and Aranda, 2001). Ligand-activated RARβ2 also stimulates neurite outgrowth of NF200 and CGRP expressing neurons in the absence of NGF. During neurite outgrowth, there is a transcriptional switch from NGF dependent where the neurites are short to NGF independent where the neurites are longer (Smith and Skene, 1997). Activated RARβ2 signalling may represent this switch as the neurites are longer in the presence of RA than those obtained in the presence of NGF alone. One of the downstream targets of RARβ2 signalling is Shh. This cannot stimulate neurite outgrowth alone but does potentiate RARβ2-mediated signalling, possibly by acting as a cell survival factor (Akazawa et al., 2004;Traiffort et al., 1999) or chemoattractant (Charron et al., 2003). The GDNF signalling pathway that leads to IB4 neurite outgrowth has little or no interaction with the RARβ2 signalling pathway.

switch is thought to take place (Smith and Skene, 1997), involves long axon extension and is neurotrophin independent because it still occurs in the absence of NGF signalling (Patel et al., 2000). RARβ2 signalling may be part of the transcriptional switch as in vitro, the length of neurites in response to RA alone is greater than in the presence of NGF. Moreover, in a peripheral nerve crush model, there is significant difference in the outgrowth of DRG axons between the RARβ2 null and wild-type mice. This suggests that in addition to cross talk with the NGF pathway, RARβ2 can also act independently to induce neurite outgrowth (Fig. 7).

RARβ regulates Shh expression which as an indirect effect on neurite outgrowth

We have shown that Shh is induced in DRG neurons in response to peripheral nerve injury, which is consistent with findings of increased Shh expression in axotomised facial motoneurons (Akazawa et al., 2004). During development, Shh signalling has been implicated in the correct development/maturation of DRG because in its absence the correct organisation of the nerve into bundles is disrupted (Powles et al., 2004), this suggests that Shh may be carrying out a similar role in DRG regeneration. The increase in Shh expression depends in part on RARβ2 expression. In the RARβ null mice, the level of Shh expression in the peripheral nerve crush is lower than in the wild-type mouse and overexpression of RARβ2 in vivo in adult motoneurons that do not normally express this receptor (Corcoran et al., 2002) increases Shh expression. This may be a direct effect as the Shh promotor contains an RARE (Chang et al., 1997).

Although Shh alone cannot induce neurite outgrowth (Fig. 7), it does enhance it in the presence of RARβ2 signalling. Inhibiting Shh signalling by cyclopamine does not prevent RA-induced outgrowth, suggesting that RARβ2 signalling regulates other neurite outgrowth pathways. Shh could potentiate RA-mediated outgrowth in at least two ways. Firstly, it may act as a neuroprotective agent (Akazawa et al., 2004; Traiffort et al., 1999); secondly, it may attract the growth of the regenerating axons from the DRG, which has previously been shown to be the case for commissural axons (Charron et al., 2003).

In conclusion, we have shown that RA signalling via RARβ2 plays a role in neurite outgrowth in vitro and in vivo and can induce Shh in the injured neuronal bodies. The further delineation of the RARβ2 pathway may allow new strategies to be developed for the treatment of nerve injuries.

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